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Inhibition of proton transfer in cytochrome *c* oxidase by zinc ions: delayed proton uptake during oxygen reduction

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Abstract

We have investigated the effect of Zn ions on proton-transfer reactions in cytochrome *c* oxidase. In the absence of Zn^{2+} the transition from the ''peroxy'' (P_R) to the ''ferryl'' (F) intermediate has a time constant of ~100 μs and it is associated with proton transfer from the bulk solution with an intrinsic time constant of ≪100 μs, but rate limited by the $P_R \rightarrow F$ transition. While in the presence of 100 μM Zn^{2+} the $P_R \rightarrow F$ transition was slowed by a factor of ~2, proton uptake from the bulk solution was impaired to a much greater extent. Instead, about two protons (one proton in the absence of Zn^{2+}) were taken up during the next reaction step, i.e. the decay of F to the oxidized (O) enzyme with a time constant of ~2.5 ms. Thus, the results show that there is one proton available within the enzyme that can be used for oxygen reduction and confirm our previous observation that F can be formed without proton uptake from the bulk solution. No effect of Zn^{2+} was observed with a mutant enzyme in which Asp(I-132), at the entry point of the D-pathway, was replaced by its non-protonatable analogue Asn. In addition, no effect of Zn^{2+} was observed on the $F \rightarrow O$ transition rate when measured in D_2O, because in D_2O, the transition is internally slowed to ~10 ms, which is already slower than with bound Zn^{2+}. Together with earlier results showing that both the $P_R \rightarrow F$ and $F \rightarrow O$ transitions are associated with proton uptake through the D-pathway, the results from this study indicate that Zn^{2+} binds to and blocks the entrance of the D-pathway.

Keywords: Electron transfer; Flow-flash; Proton pumping; Cytochrome aa_3; Flash photolysis; *Rhodobacter sphaeroides*

1. Introduction

Cytochrome *c* oxidase is located in the mitochondrial inner membrane of eukaryotes or the cell membrane of many prokaryotes where it serves as the terminal component of the respiratory chain. It catalyses the four-electron reduction of oxygen to water and couples this reaction to pumping of on average one proton per electron across the membrane (for reviews on the structure and function of terminal oxidases, see Refs. [1–4]). This paper describes results from studies of the *Rhodobacter sphaeroides* cytochrome *c* oxidase. Its structure was recently determined to atomic resolution [5] and it is very similar to those of cytochrome *c* oxidases from bovine heart [6] and from *Paracoccus denitrificans* [7].

Cytochrome *c* oxidase from *R. sphaeroides* has four subunits. Subunit I holds three redox-active metal sites, hemes *a* and a_3 and Cu_B, and subunit II holds a dinuclear Cu_A site. During turnover, electrons are transferred from a water-soluble cytochrome *c* to Cu_A and then consecutively to heme *a* and to the binuclear center consisting of heme a_3 and Cu_B where oxygen is reduced to water. The binuclear center is located approximately halfway through the mem-

Abbreviations: WT, wild-type; Cu_A, copper A; Cu_B, copper B; binuclear center, heme a_3 and Cu_B; catalytic site, the binuclear center and redox-active/protonatable groups in its immediate vicinity; N-side, negative side of the membrane; P-side, positive side of the membrane; R, fully reduced binuclear center; A, ferrous-oxy intermediate; P_R, the peroxy intermediate formed at the binuclear center upon reaction of the fully reduced (''R'') cytochrome *c* oxidase with O_2; F, oxo-ferryl intermediate; O, fully oxidized binuclear center; LM, L-dodecyl-β-D-maltoside; amino acid residue and mutant-enzyme nomenclature[1]: D(I-132), an aspartic acid of subunit I at position 132; DN(I-132); replacement of D(I-132) by asparagine

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[1] If not otherwise indicated, amino acid residues are numbered according to the *R. sphaeroides* cytochrome aa_3 sequence.

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brane and is thus not in direct contact with the bulk solution. It has been shown that the protons used for water formation are taken specifically from the N-side of the enzyme. Consequently, there must be proton-transfer pathways leading from the enzyme surface to the binuclear center. In addition, since the enzyme pumps protons across the membrane, there must be a pathway leading all the way through the enzyme.

Two such proton-transfer pathways have been identified in the bacterial cytochrome *c* oxidases [5,7–9]. The so-called D-pathway starts with an aspartic acid, D(I-132), close to the surface and continues through a number of polar residues and structurally ordered water molecules to a highly conserved glutamic acid, E(I-286), about 10 Å from the binuclear center. It has been shown to be used for proton transfer to the binuclear center and proton pumping during the oxidative part (i.e. during reaction of the reduced enzyme with O_2) of the catalytic cycle (reviewed in Ref. [10]). The second pathway, called the K-pathway, is named after an essential lysine K(I-362) in the middle of the pathway. This pathway is also composed of polar residues and water molecules forming a proton-transfer chain ending at a tyrosine (Y(I-288)), close to the binuclear center.

Zinc ions have been shown to specifically inhibit proton-transfer reactions in several membrane-bound enzymes. Nicholls and Singh [11] found that Zn^{2+} inhibits bovine heart cytochrome *c* oxidase incorporated into liposomes, as also observed with the ubiquinol oxidase from *E. coli* [12]. Recent studies showed that proton translocation by cytochrome *c* oxidase from *P. denitrificans* reconstituted in vesicles was inhibited, which was suggested to be due to blocking of proton uptake through the K- and D-pathways, i.e. the inhibition was on the inside of the vesicles [13]. Mills et al. [14] found that in cytochrome *c* oxidase from *R. sphaeroides* reconstituted in vesicles Zn^{2+} inhibited the enzyme from the outside of the vesicles and showed that the Zn^{2+} inhibition is competitive with protons. This inhibition was abolished upon addition of uncouplers; indicating a conformational change of the protein upon removal of the proton gradient.

Also in several other systems such as bacterial photosynthetic reaction centers [15–17], the mitochondrial bc_1 complex [18], and voltage-gated proton channels [19] Zn ions were shown to inhibit proton transfer. In the photosynthetic reaction centers, Zn^{2+} was shown to be coordinated by His and Asp residues. This arrangement of His residues and carboxylates is also often found around the input sites of proton pathways (cf. proton-collecting antennae, [20]).

We have earlier reported that Zn^{2+} inhibits reaction steps which involve proton uptake through the D-pathway during reaction of the reduced detergent-solubilized cytochrome *c* oxidase from *R. sphaeroides* with oxygen [21]. The results from these experiments showed that the peroxy to ferryl ($P_R \rightarrow F$) and ferryl to oxidized ($F \rightarrow O$) transitions were slowed by a factor of 2–3, although with different apparent K_D values of 120 and 2.6 μM, respectively.

To further investigate the effect of Zn^{2+} on the proton-transfer reactions in cytochrome *c* oxidase, we studied proton uptake from the bulk solution during reaction of the fully reduced enzyme with oxygen in the presence of Zn^{2+}. The results show that while in the presence of Zn^{2+} the $P_R \rightarrow F$ transition at the binuclear center was slowed by a factor of ~ 2, proton uptake through the D-pathway was slowed by a factor ≫20, i.e. the formation of F was uncoupled from the proton uptake from the bulk solution, which makes it possible to investigate these reactions separately. The nature of the inhibition was examined by investigation of the effect of Zn^{2+} in D_2O and also by investigation of Zn^{2+} inhibition in the DN(I-132) mutant enzyme. The results are discussed in terms of possible Zn^{2+} binding sites and explanations for the observed effects are proposed.

2. Materials and methods

2.1. Enzyme purification and characterisation

Expression and purification of the His-tagged *R. sphaeroides* cytochrome *c* oxidase were performed as described [22,23], with the exception that in the last step, the enzyme was eluted from the Ni-affinity column with imidazole rather than with histidine. The DN(I-132) mutant enzyme was constructed as described earlier [9]. To remove traces of Ni^{2+}, 10 mM EDTA was added to the enzyme and it was repeatedly diluted and re-concentrated using Centriprep tubes (Millipore) in 100 mM Hepes–KOH (pH 7.4) and 0.1% L-dodecyl-β-D-maltoside (LM) until the EDTA concentration was below 1 μM.

2.2. Sample preparation

To minimise the risk of metal contamination, all glassware were rinsed with a dilute EDTA solution, ethanol, 0.1 M HCl and ultra pure water. The enzyme was diluted to typically 15 μM in 100 mM Hepes–KOH (pH 7.5), 57 mM KCl, 0.1% LM. For proton uptake measurements, the buffer was exchanged to 100 mM KCl, 0.1% LM by repeatedly diluting and re-concentrating the enzyme solution, followed by an adjustment of the pH to 7.5. The enzyme solution was transferred to a modified anaerobic cuvette and the electron mediator phenazine methosulfate (PMS) was added to a concentration of 0.7 μM. After exchange of air for nitrogen, the enzyme was reduced by adding 2 mM ascorbate and the carbon monoxide adduct was formed by exchanging nitrogen for CO.

2.3. Flow-flash experiments

The experimental set-up has been described in detail earlier [24]. All tubing and syringes were flushed with a dilute EDTA solution and rinsed with ultra pure water prior to use. Measurements of absorbance changes associated with

reaction of the fully reduced enzyme with oxygen were performed as described ([25,26], see also Ref. [21]). Briefly, the fully reduced enzyme–CO complex was mixed with an O_2-saturated solution to which $ZnSO_4$ had been added to a desired concentration in a flow-flash apparatus (Applied Photophysics). About 100 ms after mixing, the CO ligand was flashed off using a ~ 100 mJ, ~ 5 ns laser flash at 532 nm, (Quantel Brilliant B, Les Ulis Cedex, France) which allows O_2 to bind and initiate the reaction.

The D_2O (Cambridge Isotope Laboratories, Inc., 99.9%) sample was prepared by gel filtration on a column PD-10 (Amersham Pharmacia Biotech), pre-equilibrated with 0.1 M Hepes buffer at a pH_{obs} (see below) of 7.5, 0.1% LM in D_2O. The fully reduced CO-bound enzyme was then prepared as described above. The experiments in D_2O were done at the same pH meter reading (pH_{obs}) as those in H_2O. Due to the deuterium isotope effect on the pH glass electrode, pD is approximately $pH_{obs}+0.4$ (see Ref. [27]). The deuterium isotope effect on the pK_as of titratable groups has a similar magnitude. Therefore, at the same pH meter reading in D_2O the "protonation" state of the group is approximately the same as in H_2O. However, one should remember that at the same pH meter reading, the concentration of D^+ is lower than that of H^+. The proton uptake measurements were performed as described [28] with the difference that phenol red was present only in the oxygen solution (and not the enzyme solution). The concentration of phenol red after mixing was 40 μM.

3. Results

3.1. Reaction of the fully reduced enzyme with O_2 in the presence of Zn^{2+}

The fully reduced CO-bound enzyme was rapidly mixed with an O_2-saturated buffer solution. Approximately 100 ms after mixing, the CO ligand was flashed off with a short laser pulse, which allows O_2 to bind to the reduced binuclear center. Absorbance changes at 445, 580 and 830 nm, associated with electron-transfer reactions as well as formation and decay of oxygen intermediates during the reaction of the fully reduced enzyme with oxygen are shown in Figs. 1 and 2 (see also Ref. [29]). After photolysis of CO, oxygen binds to the reduced binuclear center with a time constant of ~ 10 μs at 1 mM O_2, forming compound A. Then the P_R intermediate is formed with a time constant of 50 μs. This event is associated with oxidation of both hemes and is seen as a decrease in absorbance at 580 nm (Fig. 1A). As reported earlier, none of these transitions was affected by the presence of Zn^{2+} [21].

With no Zn^{2+} present, the next intermediate (F) is formed with a time constant of ~ 100 μs at pH 7.5. The formation of F is seen as an increase in absorbance at

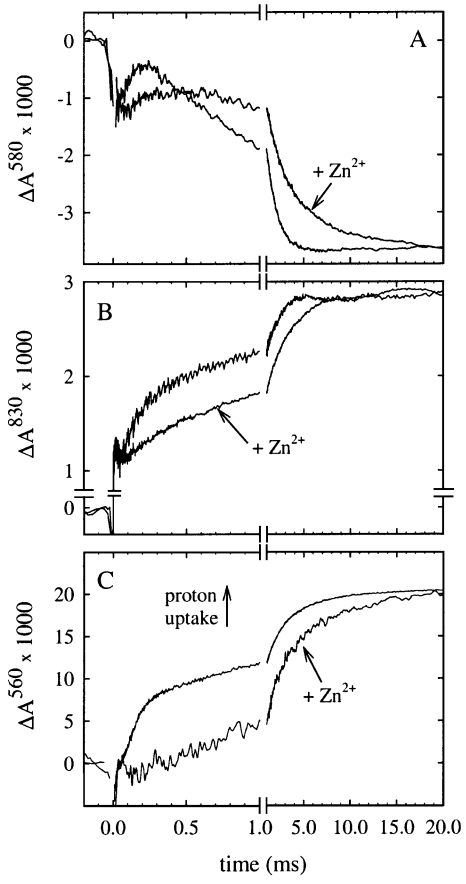

Fig. 1. Absorbance changes at 580 nm (A), 830 nm (B) and 560 nm (of the pH-dye phenol red) (C), during reaction of the fully reduced *R. sphaeroides* cytochrome *c* oxidase with oxygen with no Zn^{2+} and 100 μM Zn^{2+} present, respectively. The $P_R \rightarrow F$ ($\tau \cong 100$ μs) and $F \rightarrow O$ ($\tau \cong 1.2$ ms) transitions, seen as an absorbance increase and decrease, respectively, at 580 nm are both slowed by a factor of 2–2.5 in the presence of 100 μM Zn^{2+} (see A). With 100 μM Zn^{2+}, the electron transfer from Cu_A to heme *a* (B) as well as proton uptake from the bulk solution (C) during the $P_R \rightarrow F$ transition are impaired. Experimental conditions after mixing: (A) and (B), 0.1 M Hepes–KOH, 57 mM KCl, pH 7.5, 0.1% L-dodecyl-β-D-maltoside, 1–2 μM reacting enzyme, 1 mM O_2, $T=22$ °C; in (C), 0.1 M KCl, pH 7.5, 0.1% L-dodecyl-β-D-maltoside, 1–2 μM reacting enzyme, 1 mM O_2, 40 μM phenol red, $T=22$ °C.

580 nm (Fig. 1A) due to the formation of F itself and also due to reduction of heme *a* as a result of electron transfer from Cu_A. The latter is also seen at 830 nm (Fig. 1B) where the increase in absorbance with a time constant of ~ 100 μs is due to oxidation of Cu_A. In the presence of 100 μM Zn^{2+} the F-formation time constant increased by a factor of ~ 2.5 to ~ 250 μs (Fig. 1A). Also, the amplitude of this phase at 580 nm was smaller than without Zn^{2+} and the electron transfer from Cu_A to heme *a* was impaired (only a small absorbance change with the same rate as that of the $P_R \rightarrow F$ transition was observed at 830 nm, see Fig. 1B). In the last step, the fully oxidized enzyme is formed with a time constant of 1.2 ms. Both hemes and Cu_A are

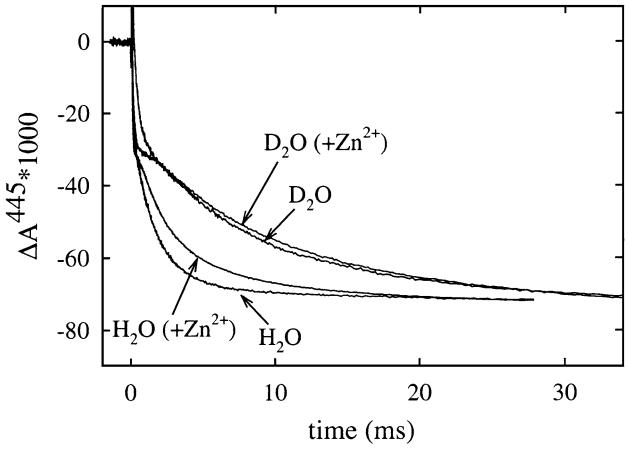

Fig. 2. Absorbance changes at 445 nm associated with the reaction of the fully reduced cytochrome *c* oxidase from *R. sphaeroides* and oxygen in H_2O and D_2O, respectively, with and without Zn^{2+}. Experimental conditions after mixing: 0.1 M Hepes–KOH, pH 7.5, 0.1% L-dodecyl-β-D-maltoside, 1–2 μM reacting enzyme, 1 mM O_2, T=22 °C.

oxidized and a proton is taken up from the bulk solution. In the presence of 100 μM Zn^{2+} this time constant increased by a factor of ~2 to approximately 2.5 ms (see Fig. 1A,B and Ref. [21]).

Without Zn^{2+}, the absorbance changes associated with proton uptake display three components, two of them being concomitant with the $P_R \rightarrow F$ (~100 μs) and $F \rightarrow O$ (1.2 ms) transitions, respectively, and one slower proton uptake that follows the formation of the oxidized enzyme ($\tau \cong 5$ ms, [25]). In the presence of 100 μM Zn^{2+}, the 100 μs proton uptake was impaired and the fastest absorbance changes associated with proton uptake displayed a time constant of 2–3 ms (it was not possible to fit the absorbance changes with a single exponential, but the average value of the two rapid components was 2–3 ms, Fig. 1C).

3.2. The effect of Zn^{2+} in D_2O

As reported earlier, the $F \rightarrow O$ transition in the *R. sphaeroides* enzyme is slowed by a factor of ~7 in D_2O ($\tau \cong 8$ ms) ([30–32], see also Ref. [33]). The addition of 100 μM Zn^{2+} did not result in any significant change in this rate measured in D_2O (Fig. 2).

3.3. The effect of Zn^{2+} in the DN(I-132) mutant enzyme

In the DN(I-132) mutant enzyme, the F-intermediate is formed with the same rate as that in the wild-type enzyme [34]. However, there is no proton uptake from the bulk solution on that time scale and the $F \rightarrow O$ transition is impaired (slowed by a factor of ~10^3, Namslauer et al., unpublished results). In contrast to the results obtained with the wild-type enzyme, no effect of Zn^{2+} (at a concentration of 100 μM) was observed on the $P_R \rightarrow F$ transition rate with the DN(I-132) mutant enzyme.

4. Discussion

We have investigated the effect of Zn^{2+} binding to cytochrome *c* oxidase on the specific steps of the catalytic reaction of the enzyme with the aim of identifying the nature of the effect and the site(s) at which Zn^{2+} binds. In an earlier study, we reported that Zn^{2+} slows the $P_R \rightarrow F$ and $F \rightarrow O$ transitions, with apparent K_D values of 120 and 2.6 μM [21], respectively, both associated with proton uptake from the bulk solution through the D-pathway.

The $P_R \rightarrow F$ transition has been shown to be composed of two distinct steps (see Fig. 3) [34,35]. First, intermediate F is formed at the binuclear center using a proton donated by an internal group, probably E(I-286). This proton-transfer reaction is rate-limiting for formation of the F-intermediate. A proton is then taken up rapidly from the bulk solution through the D-pathway to re-protonate the internal proton donor:

$$BH^+ + E(H) + P_R \xrightarrow{k_1} BH^+ + E^- + F \xrightarrow{k_2} B + E(H) + F \quad (1)$$

where BH^+ represents the bulk solution, and E(H) and E^- are the protonated and unprotonated states of E(I-286). The two events occur normally simultaneously with an observed rate of ~10^4 s^{-1} ($\tau \cong 100$ μs) because the proton uptake rate from the bulk solution through the D-pathway is $\gg 10^4$ s^{-1}, i.e. $k_2 \gg k_1 \cong 10^4$ s^{-1}.

With no Zn^{2+} added, there are two distinct proton uptake phases with time constants of ~100 μs and 1.2 ms, associated with the $P_R \rightarrow F$ and $F \rightarrow O$ transitions, respectively. Even though in the presence of 100 μM Zn^{2+}, the $P_R \rightarrow F$ transition at the binuclear center was slowed by only a factor of ~2.5 to ~250 μs, proton uptake during $P_R \rightarrow F$ was impaired and only proton uptake associated with the $F \rightarrow O$ transition was observed, which in the presence of 100 μM Zn^{2+} displays a time constant of ~2.5 ms. Considering that the time constant of proton uptake from the bulk solution that follows the $P_R \rightarrow F$ transition ($1/k_2$ in Eq. (1)) in the absence of Zn^{2+} is $\ll 100$ μs, the binding of Zn^{2+} slows the rate by a factor of $\gg 20$. Thus, the binding of Zn^{2+} to cytochrome *c* oxidase results in (i) obstruction of the proton uptake from the bulk solution and (ii) a slowing of the internal proton transfer from E(I-286) to the binuclear center by a factor of ~2. Moreover, the results from this study support our earlier proposal that the F-intermediate can be formed using an internal proton even if proton uptake from the bulk solution is blocked [34,35].

The much smaller absorbance changes at 830 nm in the presence of Zn^{2+} during the $P_R \rightarrow F$ transition indicate that there is essentially no electron transfer from Cu_A to heme *a* on this time scale ($\tau \cong 250$ μs). We have shown earlier that this electron transfer does not take place if proton uptake from the bulk solution is blocked and E(I-286) is not re-protonated [35]. Mills et al. [14] found that there is no effect of Zn^{2+} on the midpoint potential of Cu_A. Consequently, the absence of

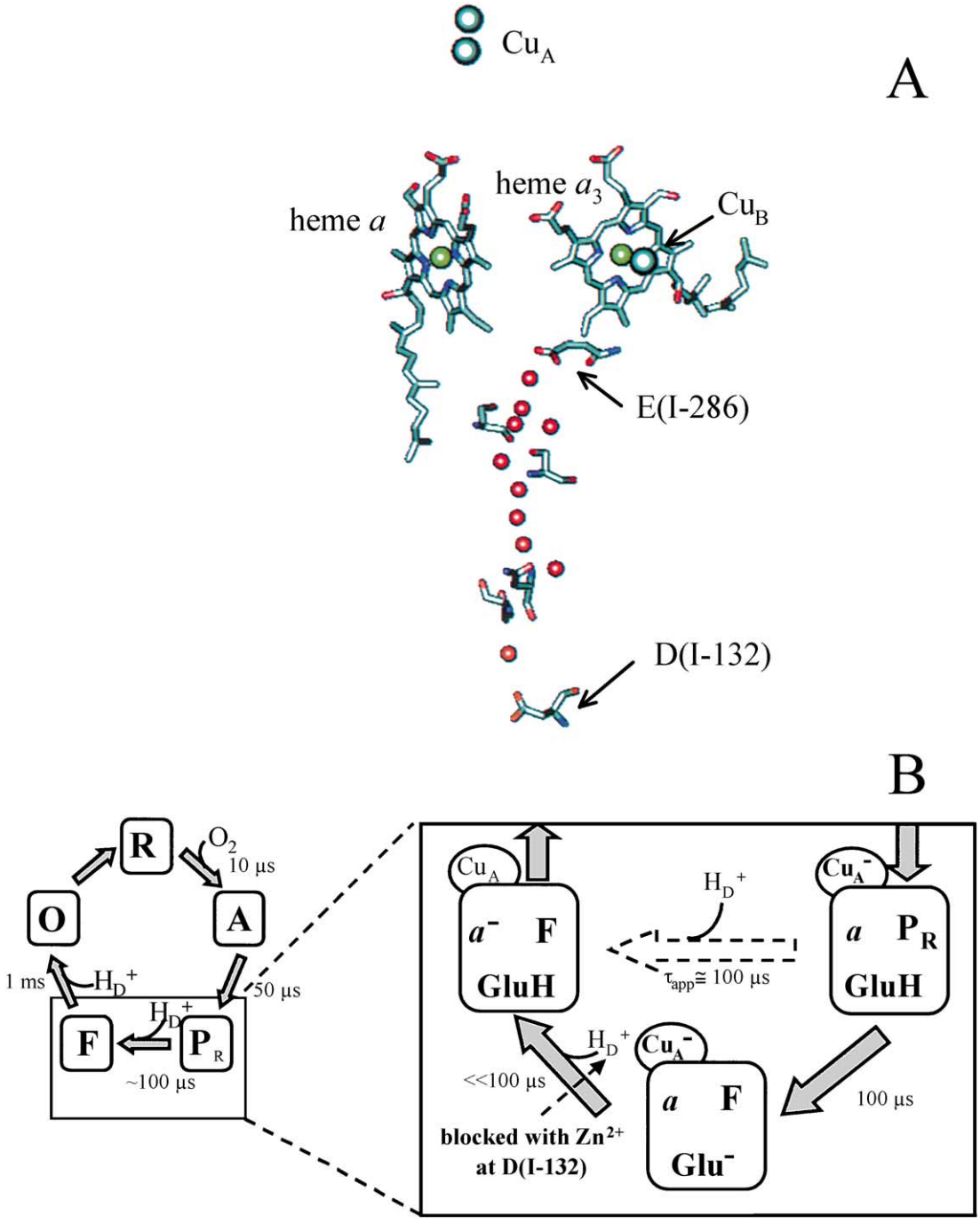

Fig. 3. (A) The D-proton-transfer pathway in *R. sphaeroides* cytochrome *c* oxidase, shown together with the redox-active sites [5]. The location of residues E(I-286) and D(I-132) discussed in this work are shown. (B) An outline of the transitions during reaction of the fully reduced enzyme with O_2. The details of the $P_R \rightarrow F$ transition are shown where the $P_R \rightarrow F$ transition is associated with *internal* proton transfer, presumably from E(I-286) to the binuclear center, followed by proton uptake from the bulk solution to re-protonate E(I-286). The latter reaction is blocked by Zn^{2+}, presumably near D(I-132). Glu^- and GluH are the unprotonated and protonated forms of E(I-286), respectively (see also text). The picture in (A) was prepared using the Visual Molecular Dynamic Software [38].

the $Cu_A \rightarrow$ heme *a* electron transfer further supports the conclusion there is an interaction between E(I-286) and heme *a*, i.e. the electron transfer does not take place because E(I-286) is not re-protonated (see Refs. [25,35,36]). As seen in Fig. 1A, the amplitude of the kinetic phase associated with the $P_R \rightarrow F$ transition at 580 nm (250 µs with Zn^{2+}) was smaller in the presence of Zn^{2+}. In the absence of Zn^{2+}, absorbance changes at this wavelength are in part associated with formation of the F-intermediate, but also in part with electron transfer from Cu_A to heme *a*. Therefore, the smaller absorb-

ance changes at 580 nm are consistent with the much lower extent of electron transfer from Cu_A to heme *a*.

The behaviour observed in the presence of Zn^{2+} resembles that found with the DN(I-132) mutant enzyme. In this mutant enzyme, the F-intermediate is formed with about the same rate as that observed with the wild-type enzyme because the proton needed to form F at the binuclear center is taken internally from the D-pathway. However, proton uptake from the bulk solution during the $P_R \rightarrow F$ transition is impaired because of the removal of the protonatable group D(I-132) at the entry point of the D-pathway. Since only the D-pathway (and not the K-pathway) is used for proton uptake during the $P_R \rightarrow F$ transition, the results with Zn^{2+} and the similarity between the effect of Zn^{2+} and the mutation of D(I-132), suggest that the Zn^{2+} ion binds near the entry point of the D-pathway, obstructing proton uptake from the bulk solution into the pathway. The binding of Zn^{2+} near the entrance of the D-pathway is further supported by the absence of any effect of addition of Zn^{2+} on the F-formation rate in the DN(I-132) mutant enzyme.

In the absence of Zn^{2+} the following transition, F→O, is associated with the transfer of one electron from the heme *a*/Cu_A equilibrium to the binuclear center and proton transfer from the bulk solution to intermediate F through the D-pathway with a time constant of 1.2 ms. Also in this step, E(I-286) acts as an internal proton donor, but the group is immediately re-protonated from the bulk solution during the proton transfer. Also, the $F \rightarrow O$ transition was slowed by a factor of ~ 2 in the presence of 100 μM Zn^{2+} ($\tau \cong 2.5$ ms). When the experiment was done in D_2O, no effect of the addition of Zn^{2+} was observed on the $F \rightarrow O$ transition rate. Earlier results showed that in D_2O, in the absence of Zn^{2+}, the $F \rightarrow O$ rate is slowed by a factor of ~ 7 ([31,32] and see Fig. 2) and the rate-limiting step was found to be within the D-pathway, presumably associated with proton transfer from E(I-286). Therefore, since the proton-transfer rate during the $F \rightarrow O$ transition in D_2O is determined by internal proton transfer and it is slower ($\tau \cong 8$ ms) than that in the presence of Zn^{2+} ($\tau \cong 2.5$ ms in H_2O), there is no effect of Zn^{2+} binding at the protein surface in D_2O, i.e. the effect of Zn^{2+} is to slow the proton-transfer rate from the outside of the enzyme into the D-pathway.

As discussed above, the $P_R \rightarrow F$ transition at the binuclear center was slowed by a factor of ~ 2.5 in the presence of Zn^{2+}. The slightly slower rate is most likely due to a slower internal proton transfer from E(I-286) in the D-pathway to the binuclear center (cf. above). The slower internal proton transfer may for example be due to electrostatic interactions between the bound Zn^{2+} and E(I-286) or due to a structural re-arrangement of water molecules in the D-pathway due to the binding of Zn^{2+} at the mouth of the pathway (cf. Ref. [17]).

A large inhibitory effect of Zn^{2+} on the proton uptake rate from the bulk solution has previously been observed with bacterial photosynthetic reaction centers where the proton-transfer rate to $Q_B^{-\cdot}$ decreased by a factor of $\gg 10^2$ in the presence of approximately stoicheiometric amounts of Zn^{2+} [15]. In the reaction centers, the binding site was identified using X-ray crystallography and the inhibitory effect was found to be due to the blocking of residues at the beginning of a proton-transfer pathway [15–17]. The Zn^{2+} was found to be coordinated by two His residues, one Asp residue and a water molecule. A similar arrangement of His residues and carboxylates is found at the entrance of the D-pathway, which makes this position a likely binding site.

On the basis of studies of the effect of Zn^{2+} binding to cytochrome *c* oxidase reconstituted in phospholipid vesicles, Kannt et al. ([13], but see Ref. [14]) found that Zn^{2+} binding on the inside of the vesicles may inhibit proton pumping. Even though our experiments were performed in detergent solution, it is apparent that the enzyme cannot pump protons during the $P_R \rightarrow F$ transition. Consequently, the results from this study explain why in the presence of Zn^{2+} the proton-pumping stoicheiometry decreases with increasing Zn^{2+} concentration [13]. Also Mills et al. [14] performed detailed studies of the Zn^{2+} effect on enzyme reconstituted in vesicles and found that Zn^{2+} added to the outside of the enzyme in coupled vesicles inhibits enzyme activity. They also found that the binding of Zn^{2+} does not compete with the binding of cytochrome *c*. The inhibitory effect was lost when uncouplers were added to the medium, indicating a conformational change resulting in alteration of the binding site. The flow-flash experiments described in this work were performed with solubilized enzyme which can be assumed to be more similar to reconstituted enzyme in uncoupled vesicles rather than in coupled vesicles. Therefore, as indicated above, we conclude that the effects seen with the solubilized enzyme originate from Zn^{2+} binding to the N-side surface of the enzyme. This conclusion is further supported by the observation of an effect of Zn^{2+} on the ND(I-139) mutant, which does not pump protons (Namslauer et al., unpublished results).

We previously found that the Zn^{2+}-inhibition of the $F \rightarrow O$ transition displays an apparent K_D value of 2.6 μM. Since the $F \rightarrow O$ transition requires proton uptake from the bulk solution (only one proton is available internally within the enzyme, see above), the lower K_D should reflect the binding of Zn^{2+} to a site near the entry point of the D-pathway.

The effect of Zn^{2+} on proton transfer in cytochrome *c* oxidase is most likely not physiologically relevant as the concentration of free Zn^{2+} in a cell is much lower than the K_D values for Zn^{2+} binding to cytochrome *c* oxidase [37]. However, the arrangement of His residues and carboxylates that has been found to constitute a typical Zn^{2+}-binding site is also a typical composition of proton-collecting antennae often found at the entry points of proton-transfer pathways [20]. Therefore, the use of Zn^{2+}

in the investigations of proton-transfer reactions in, e.g. proton pumps is a valuable tool and leads to a better understanding of the functional aspects of these systems.

5. Summary

Internal proton transfer during the $P_R \rightarrow F$ transition was slowed by a factor of ~ 2.5 in the presence of 100 μM Zn^{2+}. The effect was not observed with the DN(I-132) mutant enzyme, which indicates that Zn^{2+} binds near the D(I-132) residue.

The proton uptake rate from the bulk solution through the D-pathway was slowed by a factor of $\gg 20$ to ~ 400 s^{-1} with Zn^{2+} bound, which also determines the transition rate for the $F \rightarrow O$ reaction. The effects of Zn^{2+} on the proton uptake rate can be explained by Zn^{2+} binding close to the entry of the D-pathway, but it was difficult to discriminate between a blocking of a residue actually involved in the proton transfer and interference of Zn^{2+} with a putative proton-collecting antenna (local surface buffer).

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